Advances in Bioresearch
Adv. Biores., Vol 9 (3) May 2018: 41-46
©2018 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.9.3.4146

Advances in Bioresearch

ORIGINAL ARTICLE

Detection of *Xanthomonas campestris* pv. *cucurbitae* from Bacterial Leaf Spot Disease of Cucumber and Evaluation of Its Biological Control

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ABSTRACT
The present investigation was designed to isolate, characterization of pathogenic bacteria from leaf spot disease of cucumber (Cucumis sativus L.) and evaluation of its biological control management. Yellow pigment Xanthomonas like bacterial colonies were observed on LB agar medium after streaking and incubation at 37ºC for16 hours. Isolated bacteria were identified as Xanthomonas campestris on the basis of morphological, physiological and biochemical tests. The isolated bacterium was gram negative rod shaped and yellow in color. It showed positive result for some biochemical test like Catalase test, MacConkey test, Potassium Hydroxide test while negative result to Urease test and Kovac's oxidase test. In Triple Sugar Iron agar test and Kligler Iron Agar test the isolated bacteria fermented carbohydrates. SIM test confirms the motility of the bacteria with no indole formation and no H_2S gas production. The antibiotic and antibacterial sensitivity was determined by disc diffusion method. The antibiotic Cefotaxime revealed highest 43 mm diameter of zone of inhibition against the isolated bacteria. The extract of Allium sativum showed highest 17mm diameter of zone of inhibition against isolated bacteria. This investigation should be helpful for future detection of the pathogenic bacteria and its biological control.
Keywords: *Cucumber, Leaf spot disease, Xanthomonas campestris, Biochemical test, Biological control*

Received 02.11.2017 Revised 23.12.2017 Accepted 14.04.2018

How to cite this article:
R S Lia, Md. R Ali, Md. Saroar Jahan, A Akter, Mst. S E Sumi, Md. F Hasan, U K Acharjee, Md. A Islam and B Sikdar. Detection of *Xanthomonas campestris* pv. *cucurbitae* from Bacterial Leaf Spot Disease of Cucumber and Evaluation of Its Biological Control. Adv. Biores., Vol 9 [3] May 2018.41-46.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops, belonging to the family Cucurbitaceae. Cucumber is distributed primarily in tropical and subtropical regions of the world [1]. Different types of vegetable and fruit crops, which are related to melons, such as watermelon, cantaloupe and honeydew are a relatively low-calorie food at just about 15 calories per cup and are about 95% water. It contains high levels of lignans, vitamin K cucurbitacins and their derivatives, flavonoids, antioxidants such as beta carotene and vitamin C, and B vitamins, among other trace elements and minerals [2][3]**.** Cucumber has an important role in cooling of skin. Cucumber slices offer many benefits to the eyes and surrounding tissues through their hydrating properties, which work to reduce dehydration, their high levels of vitamin K that help reduce dark circles, and the lignans they contain for reducing inflammation [4]. Now a day, cucumbers have been used to treat wrinkles and sunburns and have been used as a moisturizer and skin brightener by inhibiting tyrosinase [5]. In Bangladesh the production of cucumber is low due to different types of disease. Different types of disease of cucumber are caused by bacteria, fungus and viruses. Bacterial diseases of cucumber are most common. In Bangladesh, bacterial leaf spot disease of cucumber is one of the most devastating disease which cause

more than 40% yield reduction [6]. *Xanthomonas campestris* pv. *cucerbitae* is the causal agent of cucumber bacterial leaf spot [7]. The symptoms of this disease is more or less similar to angular leaf spot disease of cucumber, include vein-limited, water-soaked lesions on the cucumber leaves, with or without a chlorotic halo, and water-soaked lesions on fruits, which may be misshapen [6]. The spots first appear as water soaked leisons on leaves and gradually expand until they are delimited by larger secondary veins. To overcome this problem, there is a constant need for new and effective infection fighting strategies. Therefore, there is a need to develop alternative therapeutic agents for the treatment of infectious diseases. There are few works have done in Bangladesh regarding the bacterial leaf spot disease of cucumber. Therefore, the present investigation was design to isolate bacteria from leaf spot disease of cucumber and to characterize the isolated bacteria through different types of biochemical test. Some standard antibiotic and plant extracts activities were tested against the isolated bacteria for evaluation of its biological control.

MATERIALS AND METHODS

Plant material

In the present study, disease infected cucumber plant leaves were collected from the Tiktiki village, bypass region of Rajshahi and were identified by BCSIR, Binodpur, Rajshahi, Bangladesh. Spot disease infected leaves of cucumber were used as plant material.

Isolation of bacteria

In this experiment, Bacterial samples were collected from bacterial leaf spot infected cucumber plants were cultured at Professor Joarder DNA and Chromosome Research Lab., Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh for the isolation of different single colony of bacteria on the basis of morphology. Disease infected leaves were disinfested using a dilute sodium on LB liquid media and incubated for16 hours at 37^{o}C for allow to growing bacteria. After the bacteria have grown into LB liquid medium, use a sterile loop to streak the bacteria onto a solid nutrient agar media plates and incubated for 12 hours at 37ºC.

Biochemical characterization

Gram staining: Gram staining reagents were prepared by taking crystal violet, gram's iodine, decolorizes (alcohol), and stain Safranin. Bacterial sample was smears on a slide and carefully fixed by heat. One drop crystal violet was placing on smears and holds it 1 minutes and rinse with distilled water. One drop of gram's iodine was placed on smears for 1 minutes and rinse with distilled water. Added decolorize reagents on the sample. If it is gram negative, removing the crystal violet. Finally one drop of Safranin was added for 30 seconds and rinse with distilled water and placed to air dry for several minutes. The slide was observed under 100X microscope along with one drop of immersion oil to examine shape, size, arrangement and staining reaction of bacterial isolates [6].

Motility Test: Soft agar medium was prepared in a test tube for motility test. One isolated colony was picked from the culture and inoculates the medium by stabbing the center of the medium to a depth of 1 inch [8].

Simmons Citrate Test: Citrate medium Macfaddin [9] was prepared in deionized water and sterilized at 121 *℃* for 20 minutes. After that the media was placed on a tube and cooled in a slanted position. The isolated colony was picked with a needle and the slant surface was lightly streaked. The incubation was done at 37 *℃* for 16 hours.

Catalase Test: Transferred a small amount of bacterial colony in the clean glass slide by the use of sterile loop. A drop of hydrogen peroxide was placed in the glass slide.

Potassium Hydroxide Test: In KOH test, one or two isolated colonies from the pure culture were placed on a clean slide. After that added one drop of 0.3M KOH on the top of the colonies and waited for 1 minute and observed [10].

Triple Sugar Iron Agar Test: This test was performed to determine the ability of an organism to attack a specific carbohydrate incorporated in a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulphide (H_2S) production. The TSI agar slant (long butt and short slant) containing three types of sugars (Dextrose, Lactose and Sucrose) was stabbed, streaked with inoculums and incubated at 37ºC for 16 hours.

Kligler Iron Agar Test: The medium was prepared 1 liter deionized water and sterilized at 121ºC for 20 minutes [11]. 24 hours of old culture of each isolate were stabbing the butt and streaking the surface of the tube as well as incubated aerobically at 37ºC for 16 hours.

MacConkey Agar Test: The medium were prepared by petriplates. The pH was adjusted to 7.1 and sterilized at 121 °C for 20 minutes. The medium was poured into the petri dishes and cooled to solidify

at room temperature in the laminar airflow. The isolated colony were taken with sterile loop and streaking the petri plates. The incubation was done at 37 °C for 16 hours.

Urease Test: Urease medium was prepared to added deionized water. The pH was adjusted to 6.7 and autoclaved at 121ºC for 20 minutes except urea. After autoclaved, cooled the media to 50 to 55ºC, then urea base was added into the media and mixed thoroughly. The tubes were slanted during cooling until solidified. The isolates were inoculated into the slant and incubated at 37ºC for 16 hours.

Kovac's Oxidase Test: One drop of 1% Kovacs' reagent (1gm Tetramethy-p-phenylenediamine Dihydrochloride in 100 ml distilled water) was placed on the middle of the Whitman filter paper and platinum loop full of bacterial strain was carefully rubbed on the filter paper [12].

Antibiotic sensitivity test

Antibiotic susceptibility was determined by moderate disc diffusion method [13]. The isolated bacterial strains were grown overnight in nutrient broths that were placed in the shaker at 37ºC and 150 rpm for the antibiotic sensitivity test. A serial dilution technique was made for the test respective. LB agar medium making culture plates, the sterile liquid medium was distributed in sterile conical flasks when the temperature cooled down to 40-50ºC. Approximately 15-20 ml of the medium was poured in each petridish. Commercially available and frequently prescribed antibiotics were received as antibiotic discs. 10units, 10mcg, 5µg, 15µg, 10mcg, 30mcg, 30mcg concentrations of Penicillin, Amoxycillin, Ciprofloxacin, Erythromycin, Gentamycin, Neomycine, Tetracycline, Amphicilline, Azithromycine, Carbenicilline, Kanamycine, Doxycycline, Streptomycine and Chloramphenicol respectively were used to test antibiotic sensitivity of the two isolated bacteria. Antibiotic disks were placed centrally on the respective plates and incubate at 37ºC for 16 hours.

Antibacterial activity test

Antimicrobial activity test was performed by moderate agar disc diffusion method [14,15]. Different types of plants were collected from different location of Rajshahi University campus. They were carefully washed, air-dried for 8 hours next 3 days and put in the shade in an aerated place till complete drying, then were ground into a fine powder [16]. Different plants namely, *Allium cepa, Allium sativum, Zingiber officinale, Momordica charantia, Ocimum sanctum, Phyllanthus emblica, Adhatoda vasica* and *Aloe barbadensis* were used for antibacterial activity determination against the isolated bacteria.

Statistical analysis

All the above investigations of the present study were repeated threes for consistency of results and statistical purpose. The data were expressed as meanusing Microsoft Excel 2010 version.

RESULTS

Isolation and purification

The infected leaves samples placed on LB liquid media showed the bacterial colonies after 16 hours of incubation at 37ºC. The turbid condition in the media indicates the bacteria were grown. Single colony was yellow in color. The size and shape of colonies were found to be small, medium, convex and mucoid.

Biochemical characterization

The colony of the isolate was yellow in color. The size and shape of colonies were found to be small to medium, smooth, convex and mucoid. KOH solubility test and gram staining reaction showed isolated bacterium was gram negative. In the SIM medium test no H_2S was produced no motility was found and no indole ring was formed for the isolated bacteria. After inoculating the bacteria with Hydrogen peroxide, the bubbles resulting from production of oxygen gas clearly indicate a catalase positive result for isolated bacteria. In Simon citrate test, the inoculated bacterial medium showed dark blue colour and bacteria showed positive result against citrate medium. In kovac's oxidase test isolated bacteria did not produce any purple colour so it is negative to Kovac's Oxidase test. Bacteria produced no colour round the colony in MacConkey agar, so it was no lactose fermenting. Isolated bacteria showed negative result to urease test. The responses of isolated bacteria in different biochemical test are given in the Table 1.

Antibacterial activity of some antibiotics

Cefotaxime showed the highest 43mm diameter of zone of inhibition at 100µg/disc concentration followed by Gentamycine with 25mm diameter of zone of inhibition at 10µg/disc concentration against isolated bacteria. On the other hand, Streptomycin showed the lowest 10mm diameter of zone of inhibition at 10µg/disc concentration against the isolated bacteria. The standard Azithromycin, Clarithromycin and Azithromycin showed moderate inhibition zone against the isolated bacteria. The result of antibiotic test is given in Table 2.

Antimicrobial activity of some plant extracts

The highest 20mm diameter of zone of inhibition was showed by *Momordica charantia* followed by *Allium sativum* with 17mm diameter of zone of inhibition against isolated bacteria. On the left hand, *Zingiber*

officinale showed the lowest 4mm diameter of zone of inhibition at 10µg/disc concentration against the isolated bacteria. The results are given in Table 3.

Table 1: Biochemical characteristics of *Xanthomonas campestris* pv. *cucurbitae*

Tests	Results	Optimization	Remarks
Gram staining	-ve	Small, rod shaped, pink color colony	Isolated bacteria was gram negative
Motility	+ve	Growth area extend away from the inoculation line	Isolated bacteria was motile
Simmon's citrate	+ve	Blue color	Isolated bacteria was capable to utilized citrate
Urease	-ve	No color	Isolated bacteria were not able to hydrolyzing urea to ammonia
Catalase	+ve	Oxygen bubbles	Isolated bacteria was able to produce catalase enzyme
KOH	+ve	Viscous and thread like slime	Isolated bacteria was gram negative,
TSI	+ve	Yellow color	Isolated bacteria were glucose and lactose fermenting, but no gas and H_2S was formed
KIA	+ve	Yellow color	Isolated bacteria were glucose and lactose fermenting, but no gas was formed
MacConkey agar	+ve	Pink color	Isolated bacteria were lactose fermenting
Kovac's oxidase	-ve	Colorless	Isolated bacteria gave no purple color after 60 seconds.

Table 2: Effects of some antibiotics against the isolated bacteria

Name of Antibiotic	Symbol	Disc potency (µg/disc)	Diameter of zone of inhibition (mm))	Sensitivity pattern
Amoxicillin	*AML*	10	16	Susceptible
Azithromycin	*AZM*	15	20	Susceptible
Carbenicillin	*CB*	100	17	Susceptible
Cefotaxime	*CTX*	30	43	Susceptible
Clarithromycin	*CLR*	15	12	Intermediate
Chloramphenicol	*C*	30	17	Susceptible
Gentamycin	*GEN*	10	25	Susceptible
Kanamycin	*K*	30	17	Susceptible
Streptomycin	*S*	10	10	Resistant
Tetracycline	*TE*	30	14	Intermediate

Legend: R = Resistant (5-10 mm), I = Intermediate (11-15 mm), S = Susceptible (16 mm ≥)

Table 3: Antibacterial activity of some plant extract against the isolated bacteria

Name of plant extract	**Diameter of zone of inhibition(in mm) in different dose (in µl)**			**Sensitivity pattern**
	10µl	**20µl**	**30µl**	
Allium sativum	14	15	17	Susceptible
Allium cepa	6.0	7.0	6.5	Resistant
Zingiber officinale	4.0	4.0	4.0	Resistant
Momordica charantia	14	16	20	Susceptible
Ocimum sanctum	6	6	6	Resistant
Phyllanthus emblica	6	6	7	Resistant
Terminalia arjuna	7	7.5	8.5	Resistant
Aloe barbadensis	5	6	8	Resistant
Azadirachta indica	6	6	6	Resistant
Adhatoda vasica	7	7.5	8	Resistant

Legend: R = Resistant (5-10 mm), I = Intermediate (11-15 mm), S = Susceptible (16 mm ≥)

DISCUSSION

In the present study, pathogen isolated from the bacterial leaf spot disease of cucumber showed characteristics of the genus *Xanthomonas* and confirm all the criteria for inclusion in the group of plant pathogen *Xanthomonas* [17]. The morphological characters of several isolates of *Xanthomonas campestris* pv.*cucurbitae* isolated from all over Japan was reported that all were gram negative, aerobic, non-sporing, straight rods and motile with one to five polar flagella [18]. Colonies of the isolated bacteria from bacterial leaf spot infected cucumber plant were yellow in color on nutrient agar medium. Aksoy (2006) [19] reported that the isolates of *Xanthomonas campestris* pv*. cucurbitae* were able to produce round or circular dommed shaped colonies in sucrose medium which is similar to our present findings.The angular leaf spot disease of cucumber caused by *pseudomonas syringae* pv. *lachymans*. In a gram stain test, the isolated bacterium is gram-negative bacteria which show the pink color. In potassium hydroxide (KOH) test the isolated bacteria showed viscous and form a mucoid string in 15 sec. It also indicates that the bacterium is gram gram-negative.Several biochemical tests such as gram staining, Motility test, Simmons citrate, Urease, Catalase, KOH test, TSI tests, KIA test, MacConkey agar, Kovac's oxidase test was done to characterize the *Xanthomonas campestris* as gram negative bacteria which shows pink color and rod shaped size in staining procedure [20]. The motility test which was accurately confirmed the *Xanthomonas campestris* motile [8].The *Xanthomonas campestris* pv. *cucurbetae* was able to utilized citrate and the media color change it to blue [9]. The *Xanthomonas campestris* showed negative result against the urease test which confirmed the bacteria was not able to hydrolyzing urea [21]. Vashist *et al.* (2013) [22] observed a gram negative bacteria was positive to catalase test which indicate our isolated bacteria was positive in catalase test. The *Xanthomonas campestris* formed bubbles after added hydrogen peroxide on the top of the bacterial colonies, which was confirmed our bacteria were catalase positive. In addition, Suslow *et al.* [10] performed KOH test to finally confirmed characterized gram negative bacteria of wheat, so our isolated bacteria was clearly indicates as gram negative. In TSI test, the gram negative bacteria observed positive results against these two tests [9]. *Xanthomonas campestris* bacteria turn media red to yellow. The slant and butt yellow color indicates the *Xanthomonas campestris* pv. *cucurbetae* was glucose and lactose fermenting. No H_2S was formed, because no black precipitation was found in the medium. The KIA test confirmed the *Xanthomonas campestris* was glucose and lactose fermenting but no gas was formed in the medium. The isolated bacteria turns the MacConkey agar media no pink color after overnight incubation, which indicates the *Xanthomonas campestris* was not able to ferment lactose [9]. Kovac's reagent gives no purple color after 60 seconds, which indicates the *Xanthomonas campestris* was gram negative bacterium [6]. In antibiotic test, carbenicilin showed highest 28mm diameter zone of inhibition, while amoxicillin showed the lowest 10mm inhibition zone against *Xanthomonas campestris* pv.*cucurbetae*. Antibiotic sensitivity test was helpful to find out the control measures of this disease. The zone of inhibition on a plate clearly identified the sensitivity pattern of the isolated bacteria against the different types of antibiotics [23]. In antibacterial assay, *Allium sativum* extract revealed the highest 17mm diameter of zone of inhibition and *Gingiber officinale* extract showed the lowest 4.0mm diameter of zone of inhibition against *Xanthomonas campestris* pv. *cucurbetae.* Hindi and Chabuck (2013) [16] performed antibacterial activity against some lemon extracts which evaluates the biological control of this disease. So, current investigation indicates that, the antibacterial activity depends on the species of the plants.

ACKNOWLEDGMENTS

The authors wish to thank Professor Joarder DNA and Chromosome Research Lab, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, for providing financial support and other facilities during the whole research work.

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